The E2F activators are implicated in a cell survival requirement

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Abstract

The pRB family, which includes p107 and p130 are known to control cell proliferation and differentiation (Mulligan and Jacks 1998). Their interactions with specific E2F transcription factors play a critical role in regulating the cell cycle progression. Wu et al, 2001 has shown that concomitant deletion of E2F1, E2F2, and E2F3 in Mouse Embryonic Fibroblasts (MEFs) renders them unable to proliferate. Since E2F1-3 have been implicated in driving expression of genes that promote the cell cycle, it was suggested that the inability of these cells to proliferate was due to a loss of gene expression. This *in vitro* analysis established that E2F3 must work alongside E2F1 and E2F2 to maintain normal proliferation. However, this conclusion had not been verified in differing cell types, and the *in vivo* importance of these conclusions was unknown. We show in this study that E2F1-3 are not required for cell proliferation or for differentiation of most cell types. Embryonic stem (ES) cells triply deficient for the E2F1-3 are able to proliferate, and teratomas formed from these cells are able to grow and differentiate. *In vivo* analysis using embryos triply deficient for E2F1-3 shows that they are able to survive until E9.5, suggesting that the activators are not essential during early embryogenesis. They are, however, critical for later stages of development. Similarly analysis of embryonic lenses deleted for E2F1-3 indicate their importance in particular tissues and highlights their potential role in cell cycle checkpoint regulation. The current study suggests a requirement for E2F1-3 in cell survival during development and begins to redefine the roles of E2Fs *in vivo.*

Introduction

Cellular proliferation, or cell division, is required for growth during development and tissue maintenance during adult life. Unchecked proliferation, however, can cause cancer which is characterized by populations of cells that proliferate outside normal constraints. Thus, tight regulation of the cell cycle is required for maintaining health. The *Retinoblastoma* (*RB*) gene was first identified as a tumor suppressor by its role in preventing the ocular pediatric cancer for which the gene is named. *RB*-heterozygous individuals are at high risk for developing retinoblastoma, and loss of *RB* has since been associated with many human cancers (Clarke et al. 1992; Lee et al. 1992; Maandag et al. 1994). Extensive research in both humans and mice has been done to understand the mechanisms by which cell cycle progression is regulated by Rb. The pRb family, which includes p107 and p130 are known to control cell proliferation and differentiation through their interactions with specific E2F transcription factors (Mulligan and Jacks 1998). The E2F family can be divided into four subclasses. The transcriptional activators, E2F1-3, are presumed to manage apoptosis and cell proliferation by both activation and repair of genes (Johnson et al. 1993). The second class, E2F4 and 5 are thought to maintain a repressive state. E2F1-5 share a pocket protein binding domain which allows them to interact with the pocket proteins (Müller et al. 1997). E2F6 through 8 are also considered transcriptional repressors. E2F6, the only known member of its group, forms complexes with polycomb-group proteins (PcG), while E2F7 and 8 are members of last classification and, unlike all other E2F family members, lack a dimerization partner (DP)-binding domain and function independently of it (Atwooll et al. 2004).

The classical mechanism by which the E2F family is presumed to function is characterized by presence of E2F4/p130 complexes on gene promoters in G_0. As cells begin to enter the cell cycle at the G_1/S transition, the E2F4/p130 complex leaves and is replaced by E2F/pRb. At this point, activated cyclin-dependent kinases (cdks) phosphorylate the Rb pocket proteins, which allow the E2F activators to dissociate, target genes are expressed and the cell cycle proceeds through S phase (Atwooll et al. 2004).

Traditional knockout methods in mice have been used to identify the specific functions of E2F family members. In mammals and higher eukaryotes, functional redundancy may allow the loss of one E2F to be compensated by the role of another. This has made it difficult to analyze functional specificity among family members (DeGregori 2002). In order to overcome these limitations, some investigators have knocked out combinations of genes to test for group functionality. Wu et al. (2001) tested for functional redundancy among the activator group and has shown that concurrent deletion of E2F1 and E2F3 or E2F2 and E2F3 results in embryonic lethality. This is in contrast to deletion of E2F1 and E2F2, which produces viable mice. This study pointed at a critical role for E2F3 in development. Furthermore, Wu et al., produced mouse embryonic fibroblasts (MEFs) from mice that carried a conditional allele for *E2f3*. This conditional allele allowed them to selectively eliminate *E2f3* from the genome. With introduction of a retrovirus that expressed Cre recombinase, the LoxP-flanked (floxed) *E2f3* alleles were excised from the genome. By combining the conditional allele of *E2f3* with *E2f1*- and *E2f2*-null alleles, Wu et al., generated MEFs triply deficient for the E2F activators. While MEFs deficient for E2F1 and E2F2 proliferated rapidly, those deficient for E2F1 and E2F3 and those deficient for E2F2 and E2F3 proliferated at a reduced rate. Subsequent

analysis demonstrated that triply-deficient MEFs were unable to proliferate. Since activator E2Fs have been implicated in driving expression of genes that promote the cell cycle, it was suggested that the inability of the cells to progress through the cell cycle was due to a loss of gene expression. This *in vitro* analysis established that E2F3 must work alongside E2F1 and E2F2 to maintain normal proliferation (Wu et al. 2001).

In stark contrast to the findings of Wu et al. and numerous other studies that highlight the central role that E2F plays in activation of G_1/S phase-specific genes, we show in the current study that E2F activators are not required for cell proliferation or for differentiation of most cell types. Embryonic stem (ES) cells triply deficient for the E2F activators are able to proliferate, and teratomas formed from these cells are able to grow and differentiate. *In vivo* analysis using embryos triply deficient for the activators shows that they are able to survive until E 9.5, suggesting that the activators are not essential during early embryogenesis. Furthermore, analysis of the embryonic lens facilitated a more careful investigation of cell cycle progression and differentiation in a highly organized organ *in vivo*. This study suggests that the current paradigm of how E2F regulates the cell cycle must be redefined and points to the critical role that E2F1-3 play in the protection of cell survival *in vivo*.

Materials and Methods

Mouse Strains and Breeding

The *E2f1*, *E2f2*, *E2f3*, and Crystallin-cre (Cry-cre) transgenic mice used in this study were of mixed background C57B1/6-129/SV-FVB. Mice that were $E2f1^{+/-}$; $E2f2^{-/-}$;$E2f3^{+/-}$ as determined by PCR analysis were interbred for embryos triply deficient for

the E2F activators. Embryos were collected at E6.5, E8.5, E9.5, and E11.5 and either fixed in 10% buffered formalin or frozen in OCT.

Derivation of Embryonic Stem Cells

E2f1$^{+/-}$; *E2f2*$^{-/-}$;*E2f3*$^{LoxP/LoxP}$ mice and *E2f1*$^{+/-}$; *E2f2*$^{-/-}$;*E2F3*$^{+/-}$ mice were mated and blastocysts were collected prior to implantation at E3.5. The blastocysts were cultured with gamma-irradiated primary mouse embryonic fibroblasts in 24 well plates with REGRESO medium. Embryos were re-suspended with 0.25% trypsin-EDTA. Cell colonies displaying ES cell morphology were selected and subcultured between 7-14 days post plating. *E2f1*$^{-/-}$;*E2f2*$^{-/-}$;*E2f3*$^{LoxP/-}$ colonies were determined by PCR and subsequently electroporated with Elongation Factor 1-cre plasmid. These ES cells were cultured in ES cell medium (15% FBS-DMEM, 6 mM glutamine, 0.1 mM MEM non-essential amino acids, 0.1 mM 2-mercaptoethanol, and 1000-1500 units/ml LIF) for 4-6 days and screened by PCR for recombination of the *LoxP* allele. The *wild type* control used in these experiments is the TC-1 stem cell line.

Growth Curves

For cell growth experiments ES cell lines were plated at equal density of approximately 895,400 cells suspended in 11ml of ES cell medium on successive plates. The medium was changed every other day. One plate was harvested each day for four days and cells were counted using Coulter Particle Counter Z1/D to measure growth rate.

Teratoma Formation Assays

TKO and DKO ES cell lines were derived as discussed above and cultured on feeder-free gelatin-coated tissue culture plates. Two million cells suspended in DMEM medium were subcutaneously injected into four sites on aythmic nude mice. These mice

were monitored for teratoma formation. Once the teratomas were detected, they were measured every three days. Mice were harvested between 25 and 55 days post injection for analysis. Teratomas were embedded in paraffin and sectioned.

Lens Collection and Fixation

Cry-Cre transgenic mice *E2f1*$^{+/-}$*;E2f2*$^{-/-}$*; E2f3*$^{LoxP/LoxP}$ were interbred for *E2f1*$^{-/-}$*;E2f2*$^{-/-}$*; E2f3*$^{LoxP/LoxP}$ mice. Embryos were collected at E17.5 and fixed in a 10 percent buffered formalin solution. Genotypes were determined by PCR analysis. Heads were dissected and embedded in paraffin.

Histology and Special Stains

Pregnant mice were injected with bromodeoxyuridine, BrdU, 100 µg/g body weight, 2 hours before harvesting. Embryos and teratoma tissues were embedded in paraffin and sectioned or frozen in OCT and cryosectioned. Sections were stained by hematoxylin and eosin (H&E) staining for histological examination. Immunoflourescent staining for BrdU (1:50) incorporation was used to examine proliferation. Immunoflourescent staining with phopho-histone H2AX (Ser139) (1:100) antibody was used to quantify DNA double strand breaks. Subsequent slides were stained by Ki67 antibody for proliferation. Apoptosis was examined by TUNEL with ApopTag peroxidase kit. Alcian Blue 2.5 was used to screen for acid mucopolysaccharides and cartilage. Masson trichrome stain was used to distinguish collagen and smooth muscle. Toluidine blue was used to detect the presence of mast cells. Alizarian Red S stains calcium deposits and other cations and was used to detect bone formation. Oil red was used to detect neutral fats. Proliferating cell nuclear antigen Pcna and mini-chromosome-maintenance Mcm3 stains were used to characterize E2F expression in lenses. Antibodies

against alpha-, beta-, and gamma-crystallin (1:500) were used to detect protein expression in epithelial and fiber cells of the lenses. Antibodies against phosphorylated histone H3 (P-H3) (1:200) were used to monitor cell cycle entry.

Results

ES cells do not require E2F activators for proliferation

E2F activators are believed to control cell cycle entry and target gene expression, and are directly implicated in cell proliferation. Wu et al. (2001) showed that MEFs deficient for E2F1-3 are unable to proliferate. This suggested that E2F1-3 have overlapping functions in the control of proliferation. This led us to examine more closely the growth of ES cells. Because ES cells are precursor cells that proliferate rapidly and differentiate in response to external signals, it seemed that if the E2F activators were indeed essential, they would be required for entry into the cell cycle and might even impact the differentiation process.

In order to examine the role of the E2F activators in ES cells and their potential impact on proliferation, *E2f1*$^{-/-}$*;E2f2*$^{-/-}$*;E2f3*$^{-/LoxP}$ ES cell lines were derived. These were electroporated with a cre-expressing plasmid and resulted in recombination of the conditional *E2f3* allele (*E2f1*$^{-/-}$*;E2f2*$^{-/-}$*;E2f3*$^{-/\Delta}$) in 58% of the resulting clones. *E2f1*$^{-/-}$*;E2f2*$^{-/-}$*;E2f3*$^{-/LoxP}$and *E2f1*$^{-/-}$*;E2f2*$^{-/-}$*;E2f3*$^{-/\Delta}$, cell lines were plated at equal densities for comparison of growth rates. The *wild type* cell line (TC-1) which expressed normal levels of E2F1,2,3 protein was also plated for growth rate analysis (Figure A). Overall, the TC-1 cell line grew more rapidly than the double knockout and triple knockout cell lines. There was no identifiable defect with loss of all three E2Fs, as results show that the triple-knockout cells derived from one embryo grew faster than their double-knockout

counterpart.Conversely, triple-knockout cells from a second embryo grew at a slower rate than the double-knockout counterpart (Figure B). Importantly, we have found that, unlike MEFs, (Wu et al. 2001) ES cells are able to proliferate without E2F1-3.

ES cells do not require E2F activators for differentiation

E2F1-3 appeared to be dispensable for proliferation, but we wondered whether E2F1-3 might impact differentiation of ES cells. Differentiation potential was assessed by two separate assays: growth of embryoid bodies and teratoma formation. Embryonic stem cells were grown on feeder-free bacteriological culture plates to prevent cells from adhering to the plate. LIF was removed from the medium to induce the cells to differentiate and the medium was changed every other day. Fifteen days post plating the cells were analyzed, and triple knockout, double knockout, and *wild type* cell lines were found to be equally capable of forming embryoid bodies. This suggested that there was no apparent defect in the ability of triple-knockout cells to differentiate nor were E2F1-3 required for growth of these differentiated structures (Figure C).

To confirm that E2F1-3 were not required for differentiation by a second, independent assay, ES cells were subcutaneously injected into athymic nude mice to qualitatively measure differences in differentiation potential. The rate at which double knockout and triple knockout teratomas formed was almost indistinguishable and relative size to harvest was consistent across genotypes (Figure D). The *wild-type* cell line produced teratomas that grew rapidly,consistent with what was found by the ES cell growth curve assays. Between 25 and 55 days, the mice were harvested and teratomas were collected. Histological analysis of the teratomas included both standard and special stains designed to detect differences in tissue types and components. The teratomas

analyzed by H&E staining showed that triple-knockout-derived teratomas were composed of most cell lineages and tissue types typical of teratomas (Kim et al. 2007) (Figures E & F). Cartilage and bone, however, were not found in any triple knockout samples analyzed. Teratomas were further analyzed by Alcian Blue 2.5, a copper phthalocyanin basic dye that detects acid mucopolysaccharides. Absence of alcian blue-positive cells in the triple-knockout teratomas confirmed that cartilage had not formed (Figure G). Alizarian Red S, which detects calcium, was used to screen for bone formation (Figure H). Similarly, triple-knockout teratomas were the only samples lacking bone. Masson trichrome stain was used to distinguish between collagen and smooth muscle. This indicated that triple knockout teratomas contained more collagen than double-knockout and *wild-type* samples (Figure I). Toluidine Blue detected mast cells in all samples, and Oil Red O suggested that neutral fats were present in all samples.

It appeared that specific cell types might require E2F. To identify the nature of the requirement for E2F in different cell lineages, sections of teratomas were first stained by Ki67 antibody to detect cellular proliferation. Quantitative analysis suggested that epithelial and neural cells were slightly less proliferative (Figure J & K). Secondly, we sought to detect signs of programmed cell death with TUNEL staining, and found that specific cell types in triple-knockout teratomas appeared to have an apoptotic defect (Figure L). Neural and epithelial tissues had higher numbers of apoptotic cells than did other tissue types (Figure M). This suggests that there may be lineage-specific requirements for the E2F activators. Because apoptosis can be caused by accumulation of DNA damage or deregulation of the cell cycle, we checked for DNA damage by immunostaining with gamma-phosphorylated histone H2AX (γ-H2AX) antibodies. γ-

H2AX immunofluorescent staining revealed that increased numbers of cells in epithelial tissue had double-strand breaks in DNA (Figure N & O), providing a possible explanation for the increase in apoptosis found with loss of E2F1-3 in the epithelium. Further, this suggests that E2F1-3 may help to stabilize the genome or signal to DNA repair mechanisms. Taken together, these findings point to a tissue-specific requirement for E2F1-3 in the control of proliferation, cell survival, and DNA damage prevention and repair.

The E2F activators are not required in early embryonic development

To look at the *in vivo* relevance of the requirement for the E2F activators, we interbred $E2f1^{+/-};E2f2^{-/-};E2F3^{+/-}$ mice to obtain $E2f1^{-/-};E2f2^{-/-};E2F3^{-/-}$ embryos. Embryos were collected at E3.5, E6.5, E8.5, E9.5, and E11.5. One triply deficient live embryo was found at E3.5, five were found at E6.5, one at E8.5, three at E9.5, and one at E11.5. (Figure P). Embryos and associated placentas were examined histologically by H&E after sectioning. The triply-deficient embryos appeared to be of similar size and structure to littermates (Figure Q). BrdU incorporation at E6.5 indicated that embryos and extra-embryonic components were similarly proliferative. Conversely, proliferation at E9.5, as measured by staining for Ki67, showed decreased numbers of proliferating cells in myocardium of the heart. TUNEL analysis at E6.5 suggested no difference in cell death among the embryos or extra-embryonic components. However, TUNEL analysis at E9.5 recognized an increase in apoptosis and cellular defects of the labyrinth and giant cells of the placenta.Unlike what was found in teratoma epithelial tissue, γ-H2AX at E6.5 indicated that DNA damage was not present at elevated levels in early embryos. This suggests that the E2F activators are dispensable in early embryogenesis up to E9.5, and

that they are unlikely to significantly regulate cell death, proliferation, or DNA damage between E3.5-E6.5 of development (Figure R & S). Notably, giant cells of the placenta were found to be dramatically reduced in size but did express giant cell-specific markers, as measured by *in situ* hybridization with proliferin, placental lactogen I, and placental lactogen II (Figure T and unpublished observation). Here, we show that E2F1-3 are required for development of specific lineages of the mid-gestation embryo and pinpoint the time during development that E2F1-3 are important for proliferation.

Lens Cry-Cre

In order to more carefully evaluate cell cycle regulation we bred transgenic Cre-expressing mice into a *Cry-Cre; E2f1*$^{-/-}$*; E2f2*$^{-/-}$*; E2f3*$^{LoxP/LoxP}$ genetic background. The *Cry-Cre* mice were designed to specifically express Cre in the lens epithelium and fiber compartment, allowing us to delete *E2f3* only in the lens of the eye (Zhao 2004). This conditional-deletion approach was employed because the embryonic lethality that results from concurrent deletion of E2F1-3 would preclude the study of the lens and other organs of the developing embryo. The lens is a highly organized structure and serves as an ideal model for the study of cell cycle regulation and progression. The most anterior surface of the lens contains a layer of cuboidal cells. These cells are collectively referred to as the epithelial region. Proliferation occurs preferentially at the axis of the epithelial region. Proliferating epithelial cells migrate along the periphery, moving toward the posterior portion of the lens known as the fiber compartment. As cells enter the interior of the lens, they exit the cell cycle and terminally differentiate. The cells increase in size and volume and amass a large amount of crystallin proteins. As they complete terminal differentiation, cells lose their nuclei, fuse with their neighbors, and degrade membrane

bound organelles (Garcia et al. 2005). In order to confirm Cre expression, the *Cry-Cre* strain was crossed to the ROSA26 reporter line and lenses were stained for beta-galactosidase (Soriano 1999) (Figure U).

Histological analysis of *Cry-Cre; E2f1*$^{-/-}$*; E2f2*$^{-/-}$*; E2f3*$^{\Delta/\Delta}$ was used to examine cell cycle progression and differentiation. Unexpectedly, total numbers of triply-deleted epithelial cells were reduced to 65% that of control epithelium, and the cells appeared considerably larger as compared to control samples. To investigate potential differentiation defects, lenses were stained with antibodies against alpha-, beta, and gamma-crystallin proteins (Figure V). These proteins are compartment specific and triply-deleted samples expressed equally high levels of protein as controls. Alpha-crystallin is found in both the epithelial and fiber compartments. Meanwhile beta- and gamma-crystallin proteins are particular to the fiber compartment (Garcia et al. 2005). This suggests that triply-deleted lenses are able to differentiate and express genes typical of terminally differentiated lens cells.

Lenses were also analyzed for BrdU incorporation and phosphorylated histone H3 (P-H3) in order to check cell cycle progression through S and M phases. BrdU incorporation and P-H3 in lens epithelium were similar between triply-deleted and control samples (Figure W). Interestingly, 1% of all triply-deleted fiber cells still containing nuclei also incorporated BrdU. Since fiber cells are terminally differentiated they do not typically incorporate BrdU, suggesting that loss of E2F1-3 may have prevented terminal differentiation and withdrawal of the cell cycle for these cells.

Cell death was apparent in the epithelial compartment by TUNEL as early as E13.5 but is most dramatic by E16.5 (Figure X). Importantly, a concentrated region of

DNA double strand breakage as measured by γ-H2AX appeared at the transition zone where cells from the epithelial compartment migrate posteriorly into the fiber compartment (Figure Y). Positive staining in the fiber compartment was most likely a consequence of cell fusion, loss of nuclei, and break down of membrane-bound organelles typical of normal fiber cell maturation. Notably, similar to TUNEL results, H2AX staining revealed increased staining in the transition zone.

Global gene expression data suggested that E2F target gene expression might be misregulated in the triply-deleted lenses (data not shown). To assess misexpression of E2F targets, lens sections were stained with antibodies against PCNA and Mcm3, two genes known to be directly regulated by E2F. The fluorescent signals in the epithelial cells of the lenses appeared to be more intense, and several cells within the fiber compartment expressed PCNA and Mcm3. The reason for the misexpression of these E2F target genes is poorly understood, but could be due to an increase in transcription during proliferation and/or a failure to properly regulate these genes during migration and differentiation. Importantly, E2F target gene misexpression is likely to be a driving force behind, if not the primary cause of, the DNA damage and cell death found in the triply-deficient lenses.

Though lens structure appeared normal at birth, cell death and DNA damage increased just prior to birth. This led us to investigate lens structure after birth. TKO mice 21 days old had obvious phenotypic abnormalities as compared to control siblings. Unlike their siblings' spherical and transparent lenses, these lenses appeared shriveled, asymmetrical, and cloudy (Figure Z). This indicates that, although E2F1-3 may not be

required for proliferation or differentiation, they may be required for cell survival and control of cell cycle exit in a tissue-specific manner.

Discussion

The ability of cells to proliferate is a unifying characteristic that organizes life in mammals and higher organisms. Proliferating cells have highly specialized mechanisms to maintain genomic integrity. Disruption of these mechanisms can lead to uncontrollable growth, which destroys life rather than advancing it. Pluripotent ES cells are known to develop chromosomal instability when developed in culture. It is these karyotipic defects that present a complication for use in regenerative medicine (Mantel et al. 2007). Events in ES cell growth may be similar to those in tumor development which also result in chromosomal instability. In fact, there is an increasing body of literature that suggests that malignant tumors are derived from a cancer stem cell. This demonstrates that not only the development but maintenance of such tumors is carried out by cancer stem cells (Tan et al. 2006). It is the ability of these cells to self-renew and give rise to differentiated cells that makes them interesting to a cancer study. The presence of non-differentiated cells without tissue-specific markers has made it increasingly difficult to identify cancer stem cells, but it is in itself evidence for their existence (Zucchi et al. 2007). These pluripotent populations, however, are responsible for much of early embryogenesis in mammals. Since they are directly implicated in cancer, it is surprising that little is known about how these cells regulate the cell cycle.

Cancer therapies may be advanced by the knowledge of how ES cells grow and proliferate. Understanding of the cell cycle in ES cells may provide fundamental information for preparing therapies that effectively treat malignancies. The cell cycle of

ES cells is characterized by an increased S phase duration and shortened gap phases, which likely contribute to their increased rate of proliferation. Unlike somatic cells, they seem to have high cyclin-dependent kinase (cdk) activity throughout the cell cycle, while also constitutively expressing genes that are normally cell cycle regulated (Fujii-Yamamaoto et al. 2004). Because the E2F family, in association with pocket proteins, is known to regulate the cell cycle, knowledge of their role in ES cells could help us understand how they contribute to the processes of proliferation and differentiation. It is clear that ES cells act uniquely in regulating the cell cycle. The p53 tumor suppressor protein, which has been shown to induce apoptosis in many types of differentiated cells, is abundantly present in ES cells. These ES cells are able to proliferate by activating cell-cycle checkpoints and DNA repair mechanisms unlike other cells (Prost et al. 1998). It has been suggested that ES cells minimally regulate cell cycle checkpoints and thereby allow for proliferation in these circumstances (Burdon 2001). Together, these studies support the notion that increasingly strict control mechanisms are utilized as an organism develops.

The current study shows that the E2F activators are not essential for proliferation in ES cells. *In vitro* analysis shows that $E2f1^{-/-};E2f2^{-/-};E2F3^{-/-}$ ES cells are as capable of proliferating as control samples. The E2F activator subclass is also not required for differentiation in most tissues. Teratoma analysis suggests that the activators do, however, play a role in maintenance of some tissues already committed to a particular lineage. Further, the genomic integrity of some tissues could be regulated by E2F1-3. Whether directly or indirectly, loss of E2F induced DNA damage and proliferation defects in particular tissue types. *In vivo* survival of triply-deficient embryos after E9.5

indicates that the E2F activators are dispensable in early embryogenesis. Analyses of the embryonic lens and tissue from teratomas demonstrate that E2F1-3 are required in a tissue-specific manner. They may also be required for maintenance of genomic stability and activation of cell cycle checkpoints in terminally differentiated cells. ES cells and precursor cells might not maintain a tightly regulated cell cycle that allows for proliferation and differentiation without the E2F activators. The classical view of how the E2F activators regulate cell cycle progression needs to be redefined as tissue specific requirements for the E2Fs may change their role in particular differentiation states. Further analysis of precursor cells *in vivo* may provide valuable information about how the cell cycle is regulated.

References

Attwooll, C., Lazzerini Denchi, E., and Helin, K. 2004. The E2F family: specific functions and overlapping interests. *Embo J* **23**(24): 4709-4716.

Burdon, T., Smith, A., and Savatier, P. 2002. Signalling, cell cycle and pluripotency in embryonic stem cells. *Trends Cell Biol* **12**(9): 432-438.

Clarke, A.R., Maandag, E.R., van Roon, M., van der Lugt, N.M., van der Valk, M., Hooper, M.L., Berns, A., and te Riele, H. 1992. Requirement for a functional Rb-1 gene in murine development. *Nature* **359**(6393): 328-330.

DeGregori, J. 2002. The genetics of the E2F family of transcription factors: shared functions and unique roles. *Biochim Biophys Acta* **1602**(2): 131-150.

DeGregori, J. and Johnson, D.G. 2006. Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis. *Curr Mol Med* **6**(7): 739-748.

Fujii-Yamamoto, H., Kim, J.M., Arai, K., and Masai, H. 2005. Cell cycle and developmental regulations of replication factors in mouse embryonic stem cells. *J Biol Chem* **280**(13): 12976-12987.

Kim, K., Lerou, P., Yabuuchi, A., Lengerke, C., Ng, K., West, J., Kirby, A., Daly, M., and Daley, G. (2007) *Science* **315**: 482-486.

Lee, E.Y., Chang, C.Y., Hu, N., Wang, Y.C., Lai, C.C., Herrup, K., Lee, W.H., and Bradley, A. 1992. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature* **359**(6393): 288-294.

Leone, G., DeGregori, J., Yan, Z., Jakoi, L., Ishida, S., Williams, R.S., and Nevins, J.R. 1998. E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. *Genes Dev* **12**(14): 2120-2130.

Maandag, E., van der Valk, M., Vlaar, M., Feltkamp, C., O'Brien, J., van Roon, M., van der Lugt, N., Berns, A., and te Riele, H. 1994. Developmental rescue of and embryonic-lethal mutation in the retinoblastoma gene in chimeric mice. *The EMBO Journal* **13**(18): 4260-4268.

Mantel, C., Guo, Y., Ryul Lee, M., Kim, M., Han, M., Shibayama, H., Fukuda, S., Yoder, M., Pelus., Kim, K., and Broxmeyer, H. (2007) *Blood* **109**(10): 4518-4527.

Mulligan, G. and Jacks, T. 1998. The retinoblastoma gene family: cousins with overlapping interests. *Trends in Genetics* **14** 223-229.

Prost, S., Bellamy, C.O., Clarke, A.R., Wyllie, A.H., and Harrison, D.J. 1998. p53-independent DNA repair and cell cycle arrest in embryonic stem cells. *FEBS Lett* **425**(3): 499-504.

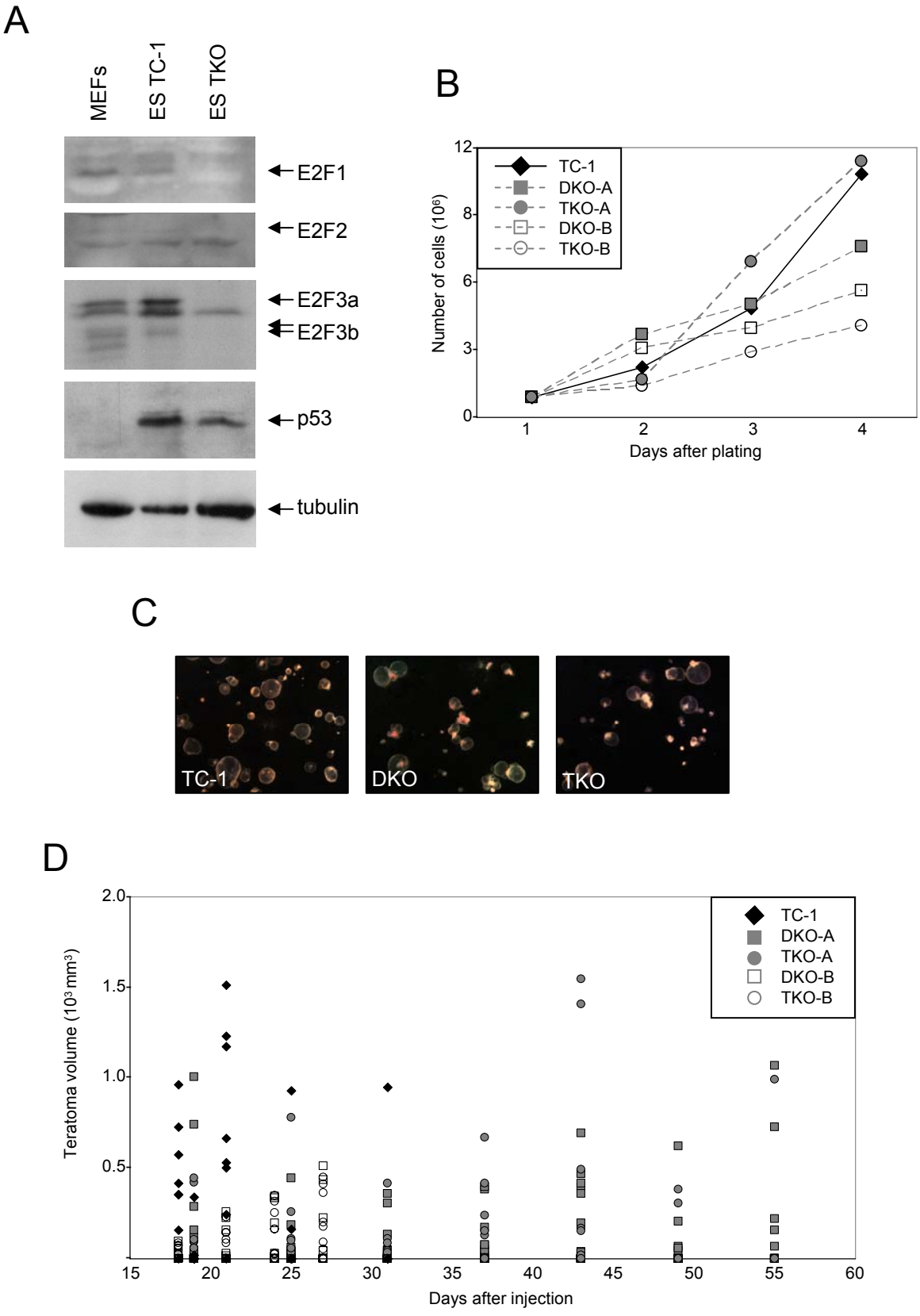
Sharma, N., Timmers, C., Trikha, P., Saavedra, H.I., Obery, A., and Leone, G. 2006. Control of the p53-p21CIP1 Axis by E2f1, E2f2, and E2f3 is essential for G1/S progression and cellular transformation. *J Biol Chem* **281**(47): 36124-36131.

Tan, B., Park, C., Ailles, L., and Weissman, I. (2006) The cancer stem cell hypothesis: a work in progress. *Laboratory Investigations* **86**: 1203-1207.

Timmers, C., Sharma, N., Opavsky, R., Maiti, B., Wu, L., Wu, J., Orringer, D., Trikha, P., Saavedra, H.I., and Leone, G. 2007. E2f1, E2f2, and E2f3 control E2F target expression and cellular proliferation via a p53-dependent negative feedback loop. *Mol Cell Biol* **27**(1): 65-78.

Wu, L., Timmers, C., Maiti, B., Saavedra, H.I., Sang, L., Chong, G.T., Nuckolls, F., Giangrande, P., Wright, F.A., Field, S.J., Greenberg, M.E., Orkin, S., Nevins, J.R., Robinson, M.L., and Leone, G. 2001. The E2F1-3 transcription factors are essential for cellular proliferation. *Nature* **414**(6862): 457-462.

Zucchi, I., Sanzone, S., Astigiano, S., Pelucchi, P., Scotti, M., Valsecchi, V., Barvieri, O., Bertoli, G., Albertini, A., Reinbold, R.A., and Dulbecco, R. (2007) The properties of a mammary gland cancer stem cell. *PNAS* **104**(25): 10476-10481.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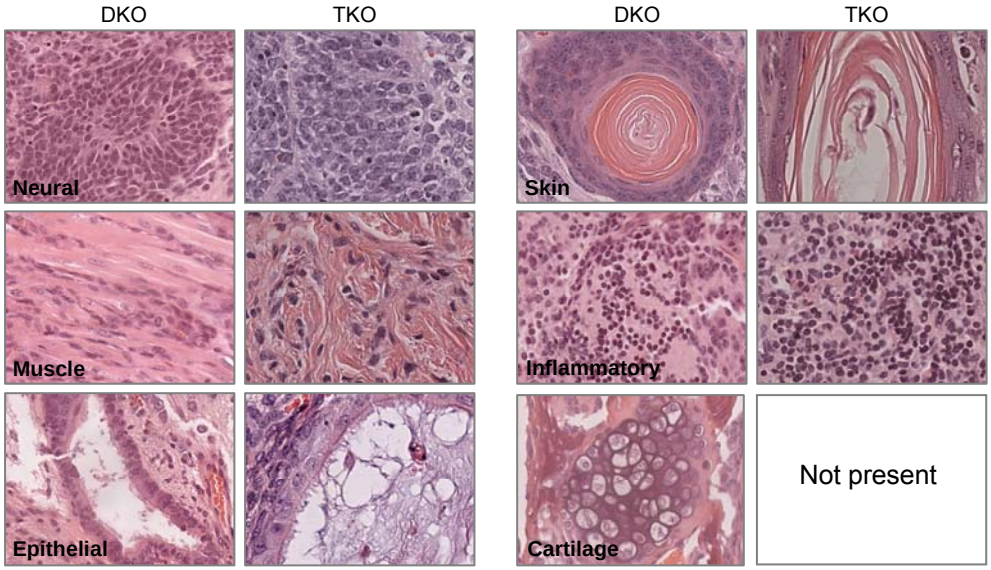
MEFs
ES TC-1
ES TKO
E2F1
E2F2
E2F3a
E2F3b
p53
tubulin
B
TC-1
DKO-A
TKO-A
DKO-B
TKO-B
Number of cells (10⁶)
Days after plating
C
TC-1
DKO
TKO
D
TC-1
DKO-A
TKO-A
DKO-B
TKO-B
Teratoma volume (10³ mm³)
Days after injection

E

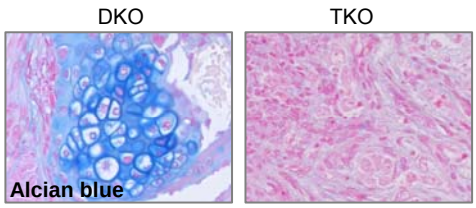


F

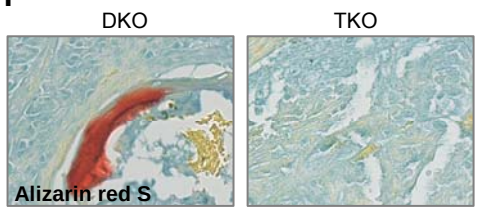
Lineages present in teratomas derived from ES cell lines

	TC-1	DKO	TKO
Neural	7	10	15
Muscle	7	11	17
Epithelial	7	12	13
Skin	1	7	3
Inflammatory	7	12	10
Cartilage	2	4	0
Total	7	12	17

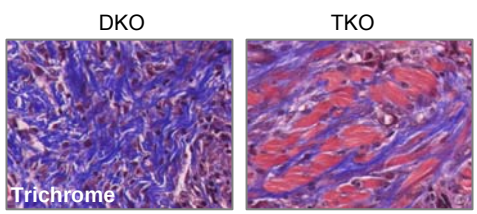
G



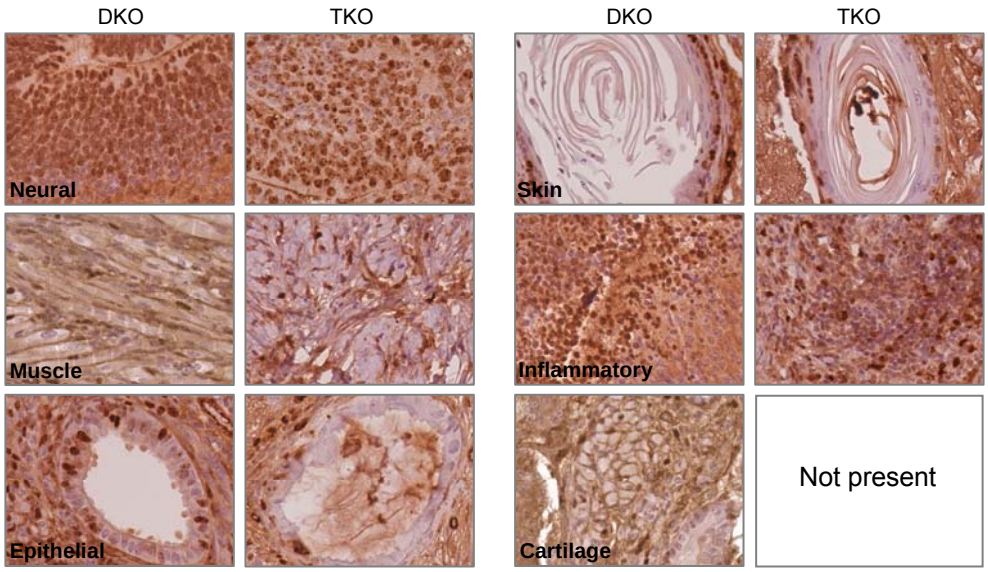
H



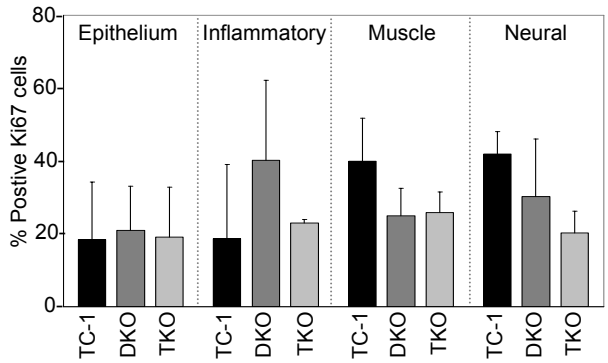
I



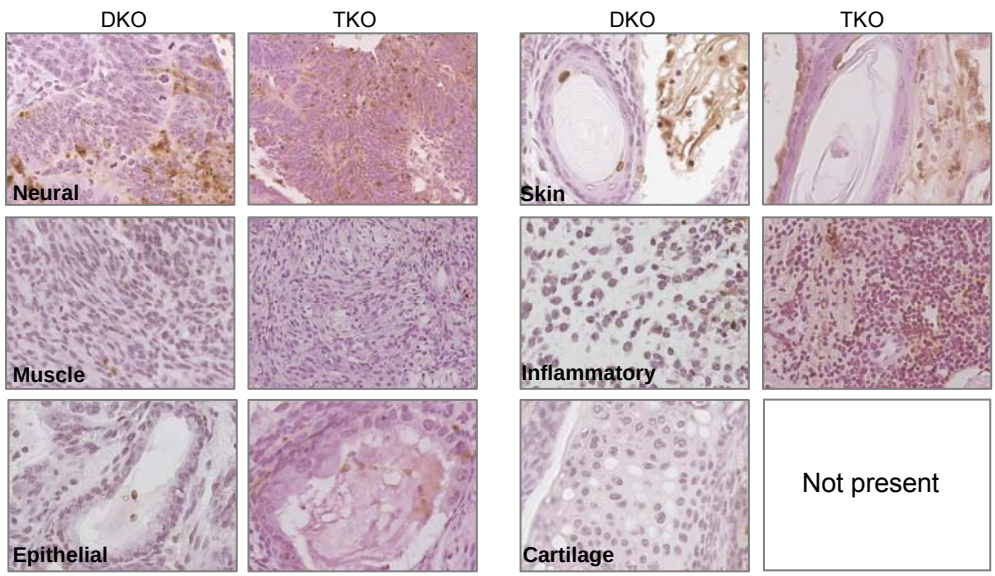
J


DKO
TKO
DKO
TKO
Neural
Skin
Muscle
Inflammatory
Epithelial
Cartilage
Not present

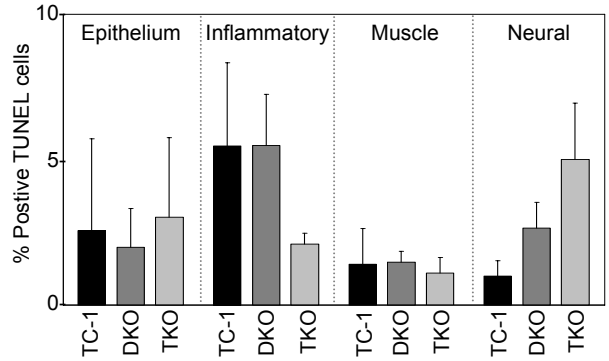
K


% Postive Ki67 cells
80
60
40
20
0
Epithelium
Inflammatory
Muscle
Neural
TC-1
DKO
TKO
TC-1
DKO
TKO
TC-1
DKO
TKO
TC-1
DKO
TKO

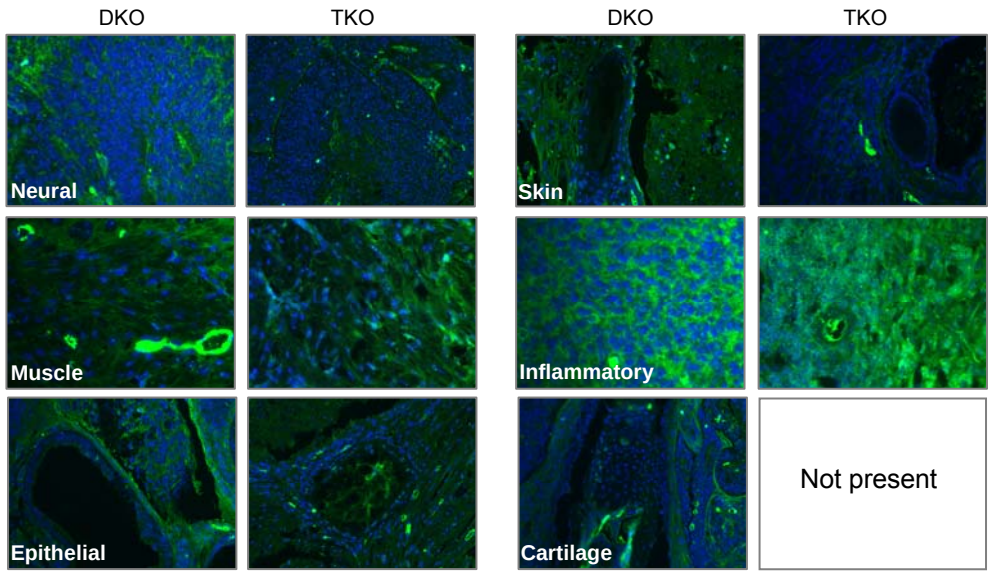
L


DKO
TKO
DKO
TKO
Neural
Skin
Muscle
Inflammatory
Epithelial
Cartilage
Not present

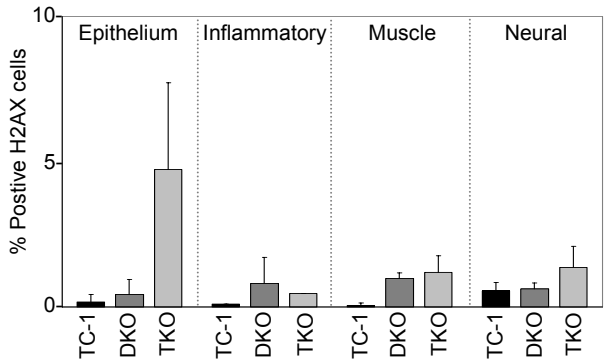
M


% Postive TUNEL cells
10
5
0
Epithelium
Inflammatory
Muscle
Neural
TC-1
DKO
TKO

N


DKO
TKO
DKO
TKO
Neural
Skin
Muscle
Inflammatory
Epithelial
Cartilage
Not present

O


% Postive H2AX cells
10
5
0
Epithelium
Inflammatory
Muscle
Neural
TC-1
DKO
TKO

P

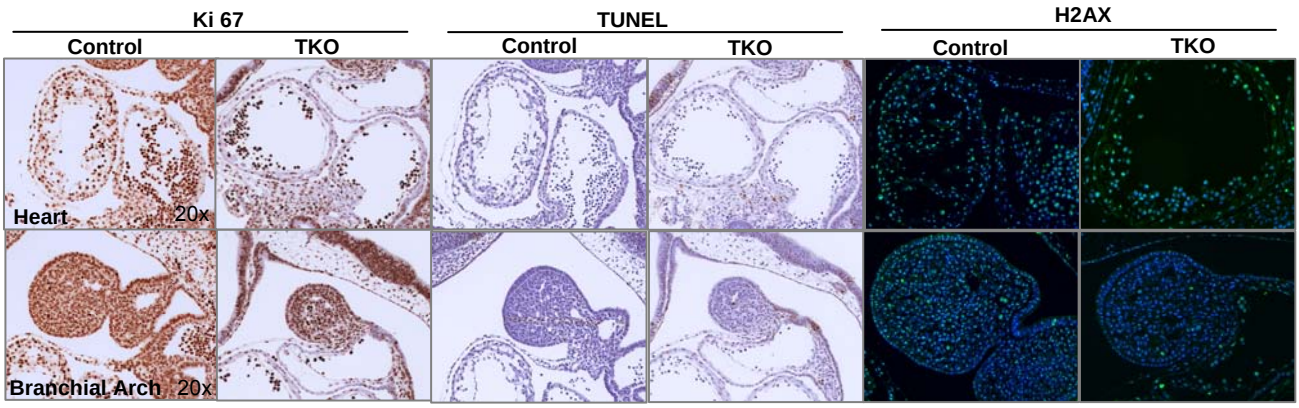
Recovery of embryos from *E2F1+/-*, *E2F2-/-*, *E2F3+/-* intercrosses

	1+/+ 2-/- 3+/+	1+/+ 2-/- 3+/-	1+/+ 2-/- 3-/-	1+/- 2-/- 3+/+	1+/- 2-/- 3+/-	1-/- 2-/- 3+/+	1-/- 2-/- 3+/-	1+/- 2-/- 3-/-	1-/- 2-/- 3-/-
E3.5	1	0	1	1	0	0	1	0	1
E6.5	0	3	0	11	41	6	7	5	5 (1)
E8.5	1	0	1	1	9	3	0	1	1
E9.5	4	3	4	10	19(1)	2	5	2	3
E11.5	2	1(1)	4(3)	4	11	2(1)	4(1)	0	1(1)

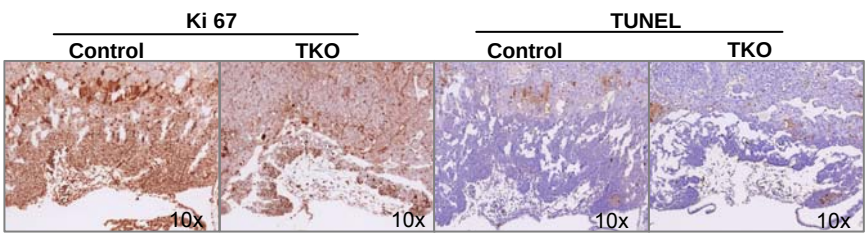
() Number of embryos dead

Q

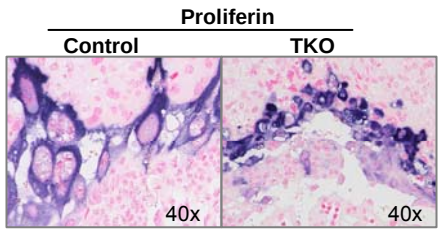
R

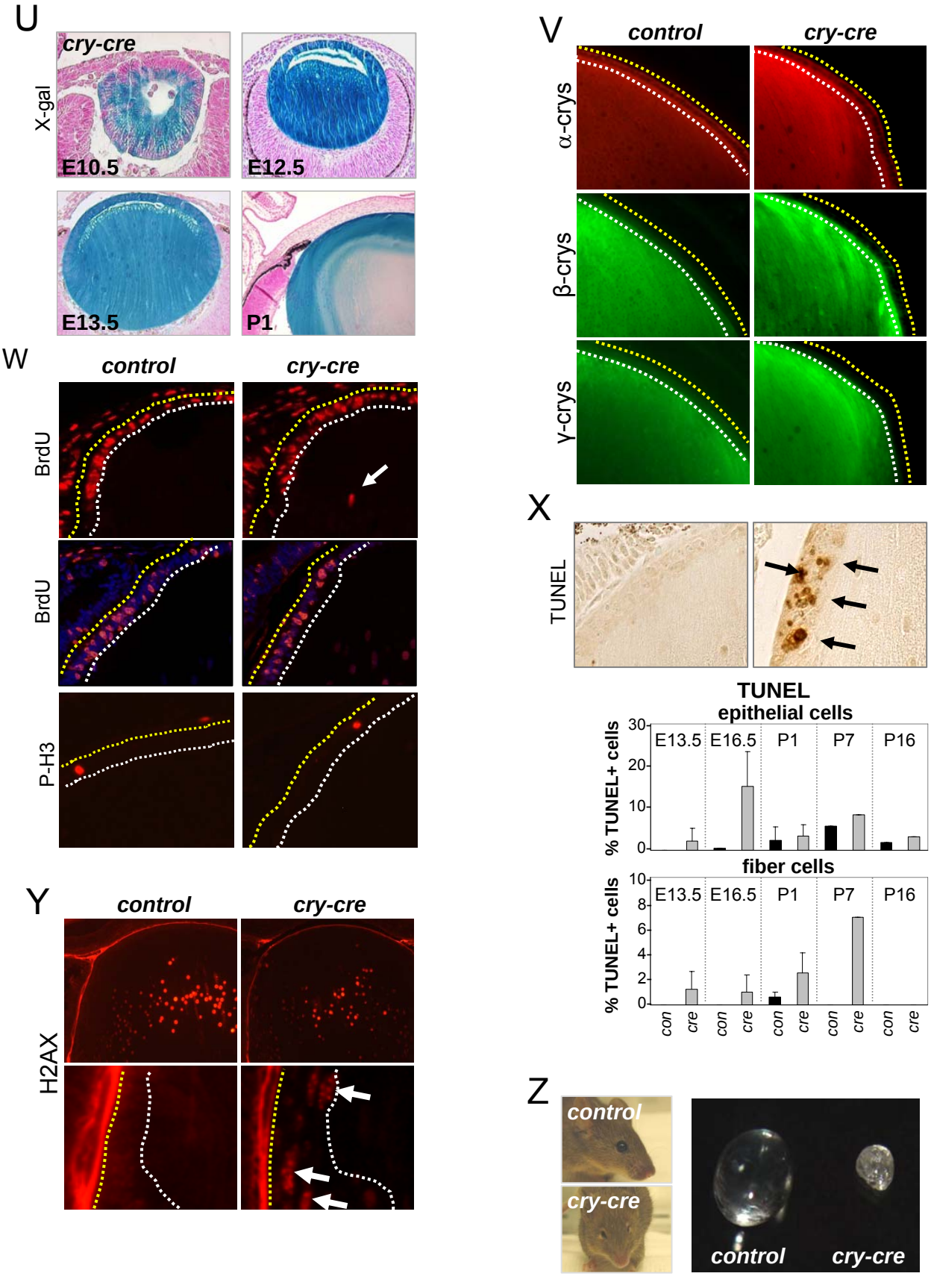


S



T




U
cry-cre
X-gal
E10.5
E12.5
E13.5
P1
V
control
cry-cre
α-crys
β-crys
γ-crys
W
control
cry-cre
BrdU
BrdU
P-H3
X
TUNEL
TUNEL
epithelial cells
% TUNEL+ cells
E13.5
E16.5
P1
P7
P16
fiber cells
con
cre
Y
control
cry-cre
H2AX
Z
control
cry-cre
control
cry-cre